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Biomass, pigment production, and nutrient uptake of *Chlorella* sp. under different photoperiods

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Abstract. *Fakhri M, Riyani E, Ekawati AW, Arifin NB, Yuniarti A, Widyawati Y, Saputra IK, Samuel PD, Arif MZ, Hariati AM. 2021. Biomass, pigment production, and nutrient uptake of* Chlorella *sp. under different photoperiods. Biodiversitas 22: 5344-5349. Chlorella* sp. is well-known as a functional feed in fish culture and has been utilized in the food industry. In phototrophic cultivation, photoperiod plays a fundamental part in the growth and pigment content of microalgae. This work was purposed to evaluate the effect of light:dark cycle on the growth rate, production of biomass and pigment and nutrient utilization of *Chlorella* sp. Four photoperiods (8:16, 12:12, 18:6, and 24:0 h light:dark regimes) under a constant light intensity of 4500 lux were applied in this study. The results demonstrated that increasing light duration led to increased cell growth, biomass, and pigment production of *Chlorella* sp. The best cell concentration, specific growth rate, and biomass production were 28.5 x 10^6 cells mL^{-1}, 1.417 day^{-1}, and 0.815 g L^{-1} dry weight, respectively, under continuous illumination. The maximum chlorophyll *a* of 19.205 mg L^{-1} and carotenoid of 4.695 mg L^{-1} were obtained at 24:0 h photoperiod. The highest uptake of nitrate (66.331%) and phosphate (76.191%) by *Chlorella* sp. were achieved under 24 h light regime. Improving the uptake of nutrients resulted in enhanced growth and pigment content of *Chlorella* sp. We conclude that continuous illumination is the best photoperiod to produce biomass and pigment and improve the nutrient removal of *Chlorella* sp.

Keywords: Carotenoid, *Chlorella*, chlorophyll *a*, growth, nitrate, phosphate

INTRODUCTION

Microalgae are microorganism that has significant advantages such as rapid growth rate (Xu et al. 2019), high efficiency for photosynthesis process (Bohutskyi and Bouwer 2013), and able to assimilate nitrogen and phosphorus compounds from wastewater (Khan and Yoshida 2008). Microalgae have attracted tremendous interest because they can be used as a feedstock for generating biofuel, feeds (Benemann 2013), and synthesizing valuable chemicals such as protein, polyunsaturated fatty acids, and carbohydrates (Pignolet et al. 2013). Moreover, due to their significant amount of pigments, some microalgae species have been applied in the industry of cosmetics, food (Khanra et al. 2018), and aquaculture (Pignolet et al. 2013). In addition, chlorophyll *a* and carotenoid are fundamental pigments for the photosynthetic process in microalgae (Johnson 2016).

The green microalga *Chlorella* sp. has been proposed as an essential feed source for farm animals and has commercially been applied in cosmetics and drugs (Sharma et al. 2012). This species produces valuable pigments such as chlorophyll (Griffiths et al. 2014), ß-carotene (Seyfabadi et al. 2011; Fathi et al. 2013), and carotenoids (Yaakob et al. 2014; Safafar et al. 2016) which makes it potential for both natural feed and nutrient supplement for aquatic animals (Safafar et al. 2016). Moreover, Ahmad et al. (2018) explained that Chlorella biomass is commercially used for feed, growth enhancer, and immunostimulant in aquaculture.

The growth rate and nutritional profile of Chlorella are affected by environmental conditions, including pH, salinity, temperature, and light (Sharma et al. 2012). Light is an elemental energy source in photoautotrophic cultivation (Wahidin et al. 2013). Light intensity, light quality, and photoperiod are known as key factors for photosynthetic activity and microalga growth (Singh and Singh 2015). Photoperiod is one of the critical conditions affecting the growth, biochemical profile, and physiological process of Chlorella (Khoeyi et al. 2012; Krzeminska et al. 2014). Chlorophyll and ß-carotene content of microalgae are also influenced by altering the light and dark cycle (Seyfabadi et al. 2011). Moreover, the duration of the light can also affect the assimilation of nutrients by microalgae (Meseck et al. 2005).

The optimization of microalga biomass is a vital step to make the microalga culture feasible for mass scale. Therefore, understanding the characteristics of microalga species under different light regimes is indispensable (Bouterfas et al. 2006) to achieve an effective growth of

Chlorella. Numerous research has been carried out to discover the influence of varying light cycle on the growth and nutritional composition of *C. vulgaris* (Seyfabadi et al. 2011; Khoeyi et al. 2012; Kendirlioglu et al. 2015; Levasseur et al. 2018). Additional information regarding how the duration of light affects the biomass and biochemical composition of *Chlorella* sp. is still interesting since microalgae's characteristics under different light conditions are strain-dependent (Banerjee et al. 2011). Moreover, the information regarding how different photoperiods influence the nutrient uptake in *Chlorella* sp. is still uncommon. This research was purposed to evaluate light and dark cycles on growth, biomass, and pigment production. In addition, this study analyzed how varying light regimes may influence the uptake of nitrate and orthophosphate by *Chlorella* sp.

MATERIALS AND METHODS

Microalgae and growth medium

The freshwater green microalga *Chlorella* sp. FNUB001 (hereafter *Chlorella* sp., Figure 1) was applied to carry out this study. It was provided by the microalga collection of Laboratory of Aquaculture, Faculty of Fisheries and Marine Science, Universitas Brawijaya, Indonesia. Cells were cultured in a modified Walne medium using sterilized freshwater (Table 1) with the axenic condition. 1 mL L^{-1} of Walne solution and vitamin B12 were added to the medium.

Experimental conditions

A 2.5 L glass bottle was applied to grow *Chlorella* sp. under batch cultivation. Four different light and dark regimes used were 08:16, 12:12, 18:06, and 24:0 light/dark. Each light/dark regime was conducted in triplicate. The cool-white tubular lamp was applied as an artificial light source (cool white fluorescence tube lamp). The light intensity of 4500 lux was exposed to all culture bottles. Aeration was given continuously by air bubbling with 1 L $minutes^{-1}$ airflow and shaken manually once a day. The temperature of 28±2ºC was kept during the experiment. Initial cell concentration and initial pH for all treatments were 5 x 10^5 cells mL^{-1} and 7.7, respectively.

Growth rate, doubling time, and biomass determination

Cell counting using a Neubauer hemocytometer (BOECO, Hamburg, Germany) was utilized to determine microalga growth (Moheimani et al. 2013; Fakhri et al. 2015; Fakhri et al. 2017). Cell counts were taken daily. The following equations were applied to analyze the specific growth rate (μ) (Sakamoto et al. 2012) and doubling time (Td) (Xu et al. 2016) of *Chlorella* sp.:

$$\mu\ (day^{-1}) = \frac{\ln (x_2) - \ln (x_1)}{t_2 - t_1}$$

$$Td\ (hours) = \ln 2/\mu \times 24$$

Table 1. Composition of Stock Modified Walne medium

Constituent	Concentration (g L^{-1})
NH_4NO_3	100.00
NaH_2PO_4	20.00
H_3BO_3	33.60
$MnCl_2.H_2O$	0.36
$FeCl_2$	1.30
EDTA	45.00
Vitamin B12	0.01

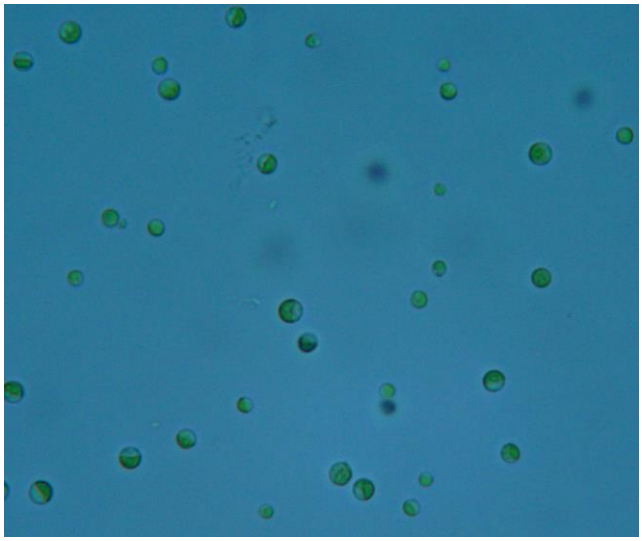

Figure 1. Morphology of *Chlorella* sp. FNUB001 cells under light microscope with 400x magnification

Biomass was analyzed as described by Janssen et al. (1999). Firstly, Whatman GF/C filter paper (diameter 9 cm) was oven at 105ºC for 2 hours and weighed until it was constant (A). On days 4 and 5 (when cells enter the stationary phase), 25 mL of culture was taken, and samples were filtered using filter paper (GF/C). Then, the algal pellet was incubated for 2 hours at 105°C and cooled in a desiccator. Finally, the dried algal and filter paper was weighed (B). Biomass concentration (dry weight, g L^{-1}) was quantified based on the following equation:

Biomass (dry weight, g L^{-1}) = (B-A) x 1000/Sample volume

Pigment determination

Microalgae at the stationary phase were used for pigment analysis. Firstly, 10 mL Chlorella suspension was centrifuged at 4032 *g*-forces (6000 rpm) for 10 minutes. For three cycles, cells were mechanically disrupted using the freezing and thawing method (frozen at -20ºC and thawed at room temperature). Then, the extract was mixed with 10 mL methanol absolute and followed by incubating at 70ºC for 30 minutes in the water bath. The solution centrifugation was done at 4032 *g*-forces for 10 min and the supernatant was kept for 24 hours at 4ºC under dark conditions. Chlorophyll *a* and carotenoid contents of

supernatant were determined as reported by Ritchie (2006) and Kim et al. (2014) methods, respectively, using a spectrophotometer (Spectroquant Pharo300) at the wavelength of 480, 652, and 665 nm.

Chlorophyll *a* ($\mu g\ mL^{-1}$) = $16.5169 \times A_{665} - 8.0962 \times A_{652}$
Carotenoid ($\mu g\ mL^{-1}$) = $4 \times A_{480}$

Nitrate and phosphate determination

Nitrate and phosphate concentrations were measured at day 0 (for all photoperiods) and day 4 (for 06:18 and 12:12 light:dark cycle) and day 5 (for 18:06 and 24:00 light:dark cycle) of culture. 80 mL alga suspension was precipitated by centrifugation at 2800 *g*-force (5000 rpm) for 20 minutes, and the filtrate was used for nitrate and phosphate analysis. Firstly, for nitrate, 12.5 mL of filtrate was poured into a porcelain dish and heated until it formed a crust. Once the mixture cooled, 0.25 mL of phenoldisulfonic acid was supplemented. Then, the mixture was diluted with 5 mL of H_2O and mixed with NH_4OH until the color changed. Next, H_2O was added to the mixture until it reached a volume of 12.5 mL. Finally, the solution was determined spectrophotometrically at 410 nm (Boyd 1979). For phosphate, briefly, 25 mL of filtrate was added to 1 mL of ammonium molybdate. Then, the mixture was added with five drops of $SnCl_2$ and homogenized. Finally, the mixture was read spectrophotometrically at 690 nm (Boyd 1979). Nitrate and phosphate concentrations were calculated from the standard curve prepared from standard nitrate and phosphate solutions, respectively.

Nutrient removal (NO_3^- or PO_4^{3-}) was analyzed by calculating the difference of nutrient concentration within a culture period (mg L^{-1}) (Osorio et al. 2020). The percentages of nutrient removal were quantified according to Acevedo et al. (2017) and Fakhri et al. (2021).

Statistical analysis

A significant difference in growth, biomass, pigment and nutrient removal among the four photoperiods was tested by ANOVA with levels of significance of 95%. SPSS v.20.0 was applied for ANOVA.

RESULTS AND DISCUSSION

Effect of photoperiod on growth and biomass production

The growth pattern of *Chlorella* sp. cultured under different light regimes was demonstrated in Figure 2. The different photoperiods of 06:12, 12:12, 18:06, and 24:00 h were performed at a light intensity of 4500 lux. Two variations of the *Chlorella* sp. growth pattern were observed under different treatments of photoperiod. Cells cultivated under 18 and 24 h light cycles reach the maximum cell concentration on day 6, while cells cultured under 6 and 12 h light cycles achieve the maximum cell concentration on day 5. It means that photoperiods with more extended periods of light (18L:6D and 24L:0D) grew for longer days than in shorter periods of light. Khoeyi et al. (2012) reported that different growth pattern was also observed under different photoperiods in *Chlorella vulgaris* culture. In contrast to our result, they found that cells grew for a longer period in 12L:12D than in 16L:8D. We suggested that different species have a specific response to various duration of light regimes.

We found a significant difference in growth rate, doubling time, and maximum cell concentration of *Chlorella* sp. under four photoperiods ($p<0.05$). The maximum cell concentration of 28.5×10^6 cells mL^{-1} and specific growth rate of 1.417 day^{-1} were observed under continuous illumination (Table 2). Table 2 also shows that increasing the light cycle led to an increase in the specific growth rate and maximum cell concentration and reduced the doubling time of *Chlorella* sp. with the shortest time of 11.776 hours under a 24-h light cycle. *Chlorella* sp. cultured under 24:0 light cycle showed approximately two times higher growth rate and 4.6 times higher maximum cell concentration than that of 06:18 light cycle. These results agreed with Xu et al. (2016), who reported the best growth rate and cell concentration were achieved under 24 h light regime. Fakhri et al. (2015) also reported that the longer microalgae were exposed to light, the higher growth rate and cell concentration were produced. We suggested that there was no photoinhibition was observed under continuous light.

The result of *Chlorella* sp. biomass production under different photoperiods is shown in Figure 3. It was noticed that light duration remarkably affected the *Chlorella* sp. biomass production. Overall, the dry weight of *Chlorella* sp. cultured in the continuous light cycle (0.815±0.022 g L^{-1}) was 78.7% higher than those cultured in 12 h light cycles (0.173±0.016 g L^{-1}). The lowest biomass concentration of cells was 0.112±0.004 g L^{-1} under six h light regime. These results are consistent with Khoeyi et al. (2012), who also found that biomass production of *C. vulgaris* increased with enhancing light duration. Similar results were also observed in Niangoran et al. (2021), who reported the biomass of *Spirulina platensis* increased with increasing light duration with the best photoperiod of 24 light regimes. Xue et al. (2011) explained that photo limitation is crucial for inhibiting microalga growth since the cells do not receive sufficient light. Singh and Singh (2015) explained that light is required to generate ATP and NADPH and produce important compounds for microalgae growth. In addition, Chauton et al. (2013) suggested low cell concentration and biomass production of microalgae under a long dark regime due to the cells not having adequate energy to promote the growth. Moreover, Jacob-Lopes et al. (2009) explained that light energy, which is received and stored in cells in the form of high-energy molecules (ATP and NADPH), is directly related to the capacity of carbon-fixation and, consequently, determining the cell metabolism and biomass concentration of microalgae.

Table 2. Specific growth rates, doubling time, and maximum cell concentrations of *Chlorella* sp. under different photoperiods

Photoperiod (L:D regime) h	Maximum specific growth rate (day^{-1})	Doubling time (hours)	Maximum cell concentration (x 10^6 cells mL^{-1})
06:18	0.740±0.027^a	22.514±0.813	6.125±0.530^a
12:12	0.867±0.010^b	19.198±0.200	9.875±0.530^b
18:06	1.324±0.030^c	12.568±0.288	22.375±0.884^c
24:00	1.417±0.089^d	11.776±0.764	28.500±0.471^d

Note: Means with different superscript letters are significantly different ($P < 0.05$)

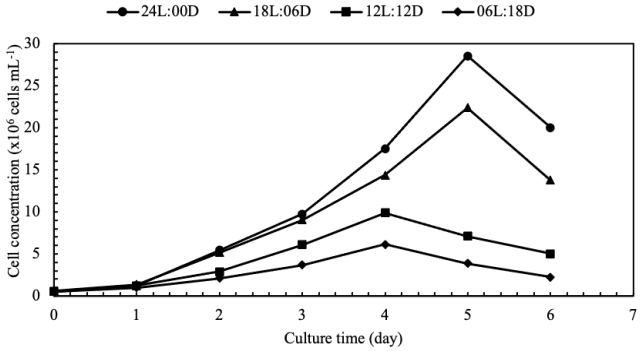

Figure 2. Cell concentration of *Chlorella* sp. under different light:dark cycles during the culture period

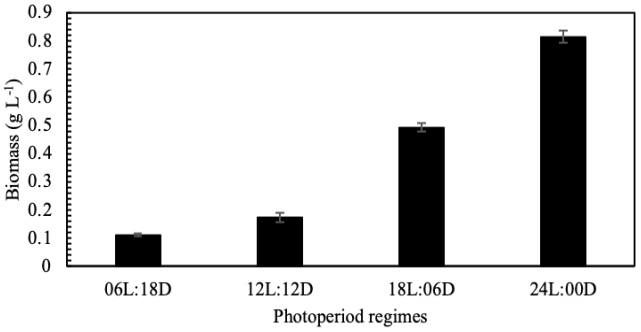

Figure 3. Biomass concentration of *Chlorella* sp. under different light:dark cycles

Effect of photoperiod on chlorophyll *a* and carotenoid content

The pigment content of cultured *Chlorella* sp. under varying photoperiods is summarized in Table 3. Cells' pigment was remarkably influenced by the light cycle ($p<0.05$). It was observed that longer light duration increased the chlorophyll *a* and carotenoid production of *Chlorella* sp. Overall, the production of chlorophyll *a* and carotenoid of *Chlorella* sp. under continuous illumination showed roughly 66% and 60% higher than that in the 12L:12D light cycle, respectively. Fakhri et al. (2017) reported that the maximum production of chlorophyll *a* and carotenoid of *Nannochloropsis* sp. BJ17 was achieved under continuous illumination. A similar profile was also observed in Fábregas et al. (2002) study, who found that the chlorophyll *a* and carotenoid production increased when *N. gaditana* was exposed to long light duration. In contrast to our results, Kendirlioglu et al. (2015) addressed that the maximum amount of chlorophyll and carotenoid of *C. vulgaris* was produced under a 16 h light regime.

In this study, the high chlorophyll *a* content under continuous illumination is probably associated with the enhancing number of photosynthetic units during light harvesting (Yusof et al. 2021). Furthermore, enhancing carotenoid content under a 24 h light regime is related to the function of carotenoids in the photosynthesis process, which is to alleviate and assist the pigment-protein complex in the thylakoid membrane (Takaichi 2011; Yusof et al. 2021). Variation of photoperiods had a different effect on the pigment content of microalgae, and it is probably dependent on the adaptation of cells to the different environments (Kendirlioglu et al. 2015; Fakhri et al. 2017; Niangoran et al. 2021).

Effect of photoperiod on nitrate and phosphate uptake

In this study, initial nitrate and phosphate concentrations were 77.50 mg L^{-1} and 15.83 mg L^{-1}, respectively. The results of nitrate and phosphate uptake of *Chlorella* sp. under different light regimes are displayed in Figures 4 and 5, respectively. From these figures, we can see that increasing light duration enhanced the nutrient uptake by *Chlorella* sp. The highest nitrate removal (66.331%) was observed in the 24L:0D light cycle, while the lowest nitrate removal (33.522%) was observed in the 06L:18D light cycle. These results revealed that long light duration increased the metabolic requirement of nitrogen and phosphorus by *Chlorella* sp. A similar profile was discovered for phosphate removal, with the maximum reduction of 76.191% was obtained in the 24L:0D light cycle, while the lowest phosphate removal of 28.171% was obtained in the 06L:18D light cycle. Liu et al. (2019) reported that increasing photoperiod enhanced the nitrogen and phosphorus uptake of photosynthetic bacteria. Zhi et al. (2019) also showed that the highest nitrogen and phosphorus utilization were observed under continuous illumination.

Interestingly, the nutrient uptake is linearly correlated to growth rate, biomass concentration, and pigment content in this study. In addition, the higher cells utilized the nutrient, and the more significant cells produced the biomass and pigment. A similar phenomenon was found that increasing nutrient uptake resulted in increased growth, biomass yield, and pigment content of *Dunaliella* sp. (Fakhri et al. 2021). Yaakob et al. (2021) also observed that increasing phosphate utilization produces high microalgae growth and biomass yield.

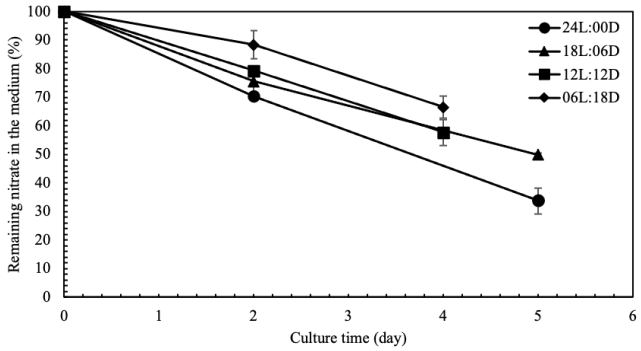

Figure 4. Nitrate uptake by *Chlorella* sp. under different photoperiods

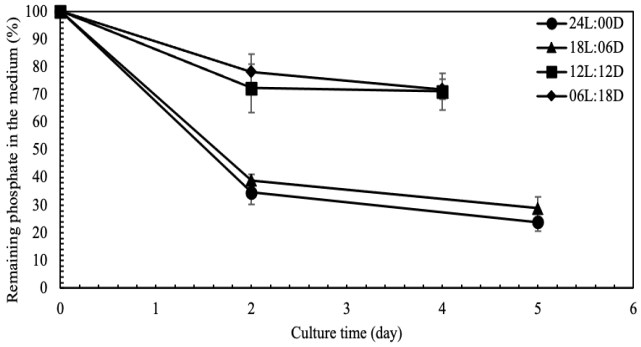

Figure 5. Phosphate uptake by *Chlorella* sp. under different photoperiods

Table 3. Chlorophyll *a* and carotenoid content of *Chlorella* sp. under different photoperiods

Photoperiod (L:D regime) h	Chlorophyll *a* (µg mL^{-1})	Carotenoid (µg mL^{-1})
06:18	2.421±0.030^{a}	0.717±0.030^{a}
12:12	6.456±0.100^{b}	1.865±0.040^{b}
18:06	13.648±0.620^{c}	3.528±0.061^{c}
24:00	19.205±1.048^{d}	4.695±0.038^{d}

Means with different superscript letters are significantly different (P<0.05)

Nitrogen plays an essential part in microalgal culture because it is the key element for proteins, chlorophylls, and DNA synthesis (Pancha et al. 2014). Zarrinmehr et al. (2020) explained that nitrogen regulates microalga growth and biomass. Besides, phosphorus is one of the major components for microalga growth and has a fundamental role in ATP production for energy metabolism (Chen and Chen 2006). The inorganic phosphate is involved in the regulation of enzyme activity, biochemical pathways, and the biological transport system, as well as influencing the photosynthesis process (Mimura 1995; Benavente-Valdés et al. 2016). Kujawinski et al. (2017) indicated that the availability of phosphorus influences intracellular nucleotide pools in microalgae and, as a result, affects their growth and biomass production.

In conclusion, it has been shown from this study that photoperiod exhibits an essential role in influencing the growth, biomass, pigment production, and nutrient removal of *Chlorella* sp. Enhancing light cycle exposure increased the growth and biomass production of *Chlorella* sp. The highest pigment content of *Chlorella* sp. was observed under continuous illumination. In addition, increasing light duration led to improving the nutrient uptake by *Chlorella* sp. When cells are exposed to a long dark regime, the cells cannot uptake the nutrient. Therefore, increasing nutrient utilization leads to higher growth, biomass, and pigment content of *Chlorella* sp.

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